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Journal

Cell transplantation, 26(10)

ISSN

0963-6897

Authors

Syu, Jia-Ning
Yang, Mei-Due
Tsai, Shu-Yao
et al.

Publication Date


2017-10-01

DOI

10.1177/0963689717724792

Peer reviewed

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Cell Transplantation
2017, Vol. 26(10) 1636–1647
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DOI: 10.1177/0963689717724792
journals.sagepub.com/home/cti


Jia-Ning Syu¹, Mei-Due Yang^{2,3,4}, Shu-Yao Tsai⁵,
En-Pei Isabel Chiang⁶, Shao-Chih Chiu^{7,8}, Che-Yi Chao^{5,9},
Raymond L. Rodriguez¹⁰, and Feng-Yao Tang¹

Abstract

Studies suggest that a low level of circulating human endothelial progenitor cells (EPCs) is a risk factor for ischemic injury and coronary artery disease (CAD). Consumption of S-allylcysteine (SAC) is known to prevent CAD. However, the protective effects of SAC on the ischemic injury are not yet clear. In this study, we examined whether SAC could improve blood flow recovery in ischemic tissues through EPC-mediated neovasculogenesis. The results demonstrate that SAC significantly enhances the neovasculogenesis of EPCs *in vitro*. The molecular mechanisms for SAC enhancement of neovasculogenesis include the activation of Akt/endothelial nitric oxide synthase signaling cascades. SAC increased the expression of c-kit, β -catenin, cyclin D1, and Cyclin-dependent kinase 4 (CDK4) proteins in EPCs. Daily intake of SAC at dosages of 0.2 and 2 mg/kg body weight significantly enhanced c-kit protein levels *in vivo*. We conclude that dietary consumption of SAC improves blood flow recovery and prevents ischemic injury by inducing neovasculogenesis in experimental models.

Keywords

S-allylcysteine, neovascularization, c-kit, Akt, human endothelial progenitor cells

Introduction

Coronary artery disease (CAD) and ischemic stroke are the most common causes of death in individuals with cardiovascular disease (CVD) worldwide.^{1,2} Due to the endothelial dysfunction, critical limb ischemia (CLI) is a major risk factor for CAD and stroke.³ Therefore, revascularization of ischemic injuries plays a key role in the tissue repair process.⁴

Previous studies have shown hematopoietic endothelial progenitor cells (EPCs) derived from bone marrow (BM) comprise a provasculogenetic subpopulation of hematopoietic stem cells (HSCs).^{5–8} EPCs are able to induce revascularization of ischemic injuries and facilitate tissue regeneration.^{9,10} Therefore, these circulating EPCs are believed to prevent ischemic injuries such as CAD and stroke with their potential for neovascularization.¹¹ These human hematopoietic EPCs and HSCs are characterized by cell surface markers, including CD 34, CD133, CD105 (endoglin), and CD309 (Flk-1/KDR). In mice, hematopoietic EPCs and HSCs bear membrane receptors such as c-kit, Sca-1, CD34, and Flk-1.^{12,13} These circulating BM-derived EPCs engraft easily, migrating into the injured tissues following ischemia, initiating neovasculogenesis, and participating

¹ Biomedical Science Laboratory, Department of Nutrition, China Medical University, Taichung, Taiwan, Republic of China

² Department of Clinical Nutrition, China Medical University Hospital, China Medical University, Taichung, Taiwan, Republic of China

³ Department of Surgery, China Medical University Hospital, China Medical University, Taichung, Taiwan, Republic of China

⁴ Department of Medicine, China Medical University, Taichung, Taiwan, Republic of China

⁵ Department of Health and Nutrition Biotechnology, Asia University, Taichung, Taiwan, Republic of China

⁶ Department of Food Science and Biotechnology, NCHU-UCD Plant and Food Biotechnology Center and Agricultural Biotechnology Center (ABC), National Chung Hsing University, Taichung, Taiwan, Republic of China

⁷ Graduate Institute of Immunology, China Medical University, Taichung, Taiwan, Republic of China

⁸ Center for Cell Therapy, China Medical University Hospital, Taichung, Taiwan, Republic of China

⁹ Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan, Republic of China

¹⁰ Department of Molecular and Cellular Biology, University of California, Davis, CA, USA

Submitted: February 4, 2016. Revised: July 2, 2017. Accepted: July 5, 2017.

Corresponding Author:

Feng-Yao Tang, Biomedical Science Laboratory, Department of Nutrition, China Medical University, 91 Hsueh-Shih Road, Taichung 40402, Taiwan, Republic of China.

Email: vincenttang@mail.cmu.edu.tw



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vascular repair.^{14,15} This insight may lead to discoveries of EPC applications that are helpful in the prevention and treatment of many diseases.¹³ This assumption is supported by a study that demonstrated therapeutic neovascularization in ischemic tissues was induced by the transplantation of EPCs.^{16,17} A clinical study showed that a reduced circulating peripheral level of EPCs is a risk factor for both CAD and stroke.¹⁸ Strong correlations were also reportedly found between the severity of atherosclerotic stress and EPC levels.¹⁹ With a cooperation of different types in many tissues, EPCs participate in the turnover of healthy and damaged endothelium in a variety of different tissues, maintaining regenerative and reparative potential, thereby playing an important role in delaying the development of atherosclerosis and CVD.⁸ This suggests that an increasing level of EPCs provides a mean for lowering the risk of ischemic injuries and CAD through the induction of neovascularization.^{20,21}

Unlike neovasculogenesis, adult angiogenesis is the formation of new blood vessels from preexisting ones, a process that requires the proliferation and migration of human microvascular endothelial cells (ECs).²² It is known that the growth of human ECs from capillary vessels contributes to postnatal angiogenesis in many physiological and pathological conditions.^{23,24} However, these mature ECs are terminally differentiated and exhibit a low level of differentiation potential.²⁵ Therefore, these adult ECs may have reduced capacity for the repair of damaged tissues.

Stem cell factor (SCF), a cellular mitogen, induces cell migration and neovasculogenesis.^{25,26} It has been suggested that c-kit protein, a receptor for SCF, is important for the blood vessel formation of EPCs.²⁵ For this reason, the c-kit receptor protein and downstream signaling molecules have attracted considerable attention for their ability to regulate neovasculogenesis.²⁷ Recent studies have suggested that SCF induces neovasculogenesis through the downstream c-kit protein and Akt signaling pathway in EPCs.^{25,28,29} This suggests a connection between the PI3-K/Akt signaling pathways and the increased capacity of EPCs to promote neovasculogenesis.³⁰ Results have also demonstrated the crucial function of Akt signaling in the migration and proliferation of EPCs.²⁹ Previous study indicated that Akt is required for the activation of the downstream endothelial nitric oxide synthase (eNOS), which increases the migration capacity in EPCs.³¹ It is also well known that the phosphorylation of the Akt protein enhances cell proliferation through phosphorylation of GSK-3 β and increased levels of the cyclin D1 protein.³²

Due to the augmentation of neovasculogenesis by dietary approaches, EPCs have attracted considerable interest from biomedical researchers and nutritional scientists. Epidemiological studies have reported an inverse correlation between the consumption of organosulfur compound-containing vegetables and the incidence of CAD.³³ The best known organosulfur compounds associated with CAD protection are contained in garlic.³⁴ The organosulfur compounds in garlic that appear to be most cardioprotective are diallyl disulfide (DADS), diallyl

trisulfide (DATS), and S-allylcysteine (SAC).³⁵ Compared to DADS and DATS, SAC is the most abundant organosulfide in the hydrophilic fraction of aged garlic extract (AGE).^{35,36} Many studies have shown that the intake of garlic components reduces the risk of CAD.³⁴ Other studies have reported that garlic consumption is associated with a reduced prevalence of ischemic injuries^{37,38} and that intake of AGE lowers the risk of CAD and stroke.^{33,39} SAC increases nitric oxide (NO) bioavailability and enhances endothelial function through its antioxidant effects.⁴⁰ It has been reported that SAC consumption effectively prevents ischemic myocardium and chronic diseases such as CAD due to its anti-inflammatory and antioxidant effects.⁴⁰

Our previous study on the oleophilic constituents of garlic extract suggested that DADS and DATS significantly induced neovasculogenesis in EPCs.³⁸ However, comparable preclinical studies of the neovasculogenesis and ischemia-protective properties of SAC in EPCs were not available. Therefore, the current study investigates the application of SAC for the simulation of neovasculogenesis and its impact on ischemic injury prevention in both in vitro and in vivo models.

Materials and Methods

Reagents

The Endothelial Cell Growth Medium-2 (EGM-2) growth kit was obtained from Lonza, Inc. (Allendale, NJ, USA). Fetal bovine serum (FBS) and protein extraction kit (Nuclear and Cytoplasmic Extraction Reagents [NE-PER] extraction kit) were purchased from Thermo Fisher Scientific, Inc. (Pittsburgh, PA, USA) and Pierce Biotechnology Inc. (Lackford, IL, USA), respectively. Antiactin antibody, SAC (purity > 99%), L-NG-Nitroarginine methyl ester (L-NAME) (a specific inhibitor of eNOS), and wortmannin (a specific inhibitor of PI3-K) were obtained from Sigma, Inc. (St Louis, MO, USA). Immun-Blot Polyvinylidene difluoride (PVDF) membrane was purchased from BioRad (Hercules, CA, USA). Anticyclin D1 monoclonal antibody, Matrigel[®], stem cell factor (SCF), and collagen were purchased from BD Bioscience, Inc. (San Jose, CA, USA). Other monoclonal antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). These antibodies were cross-reactive with phospho-Akt (p-Akt; T308), total-Akt (t-Akt), phospho-eNOS (p-eNOS; S1177), total-eNOS (t-eNOS), phospho-GSK-3 β (p-GSK-3 β ;S9), total-GSK-3 β (t-GSK-3 β), β -catenin, c-kit, CDK4, histone H3, and lamin A proteins, respectively.

Preparation of Human EPCs

All protocols conformed to the ethical guidelines of the 1975 Declaration of Helsinki in a prior approval by the institutional review board of the China Medical University Hospital. Human EPCs were generated from fresh human umbilical cord blood mononuclear cells (MNC) as described previously.⁴¹ Human EPCs produced from the method exert

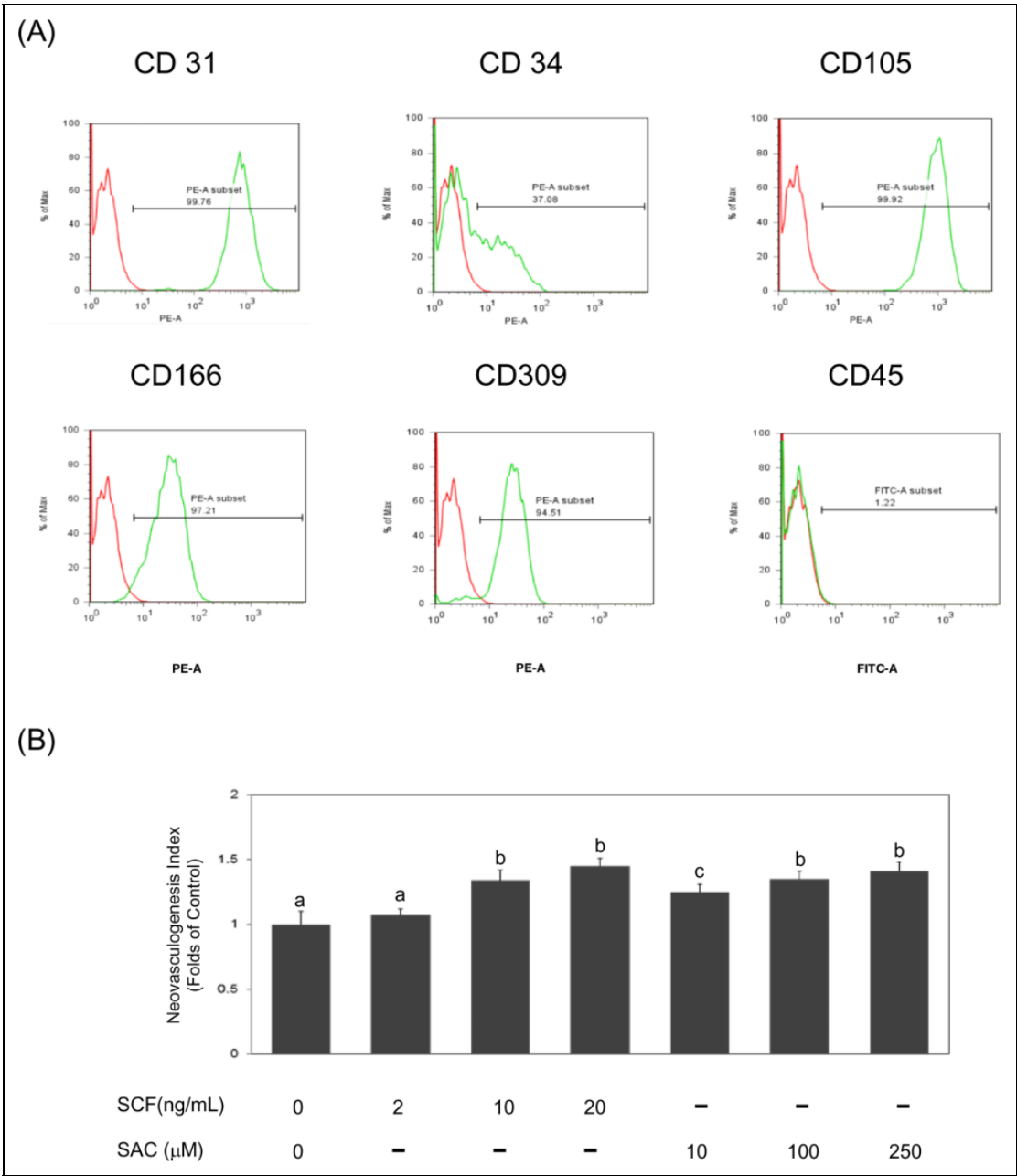


Fig. 1. S-allylcysteine (SAC) effectively induces neovascuogenesis of endothelial progenitor cells (EPCs) in vitro. Characterization of EPC-specific cell surface antigens by flow cytometry analysis (A). EPCs were in the condition of stem cell factor (2, 10, and 20 ng/mL) or SAC (at concentrations of 10, 100, and 250 μM) for 8 h until the analysis of neovascularization (B). The values, mean ± standard deviation, were represented as neovascuogenesis index (folds of tube formation in comparison with the untreated control subgroup) in 8 randomly selected fields of each culture dish. Results of each subgroup were measured in triplicate and repeated twice. A different letter indicates a statistically significant difference among different subgroups ($P < 0.05$).

a highly proliferative potential.⁴² In brief, MNCs were isolated by using Ficoll-Paque procedure. Colonies of EPCs were featured with cobblestone shape while culturing between 5 and 22 d. EPC colonies were selected and cultured in 10% FBS MCDB-131 with an EGM-2 growth kit in a collagen-coated tissue culture dish. Immunophenotyping of EPCs was analyzed by fluorescence-activated cell sorting (FACS; Becton Dickinson, San Diego, CA; Fig. 1A). We

used primary monoclonal antibodies against human CD31 conjugated to phycoerythrin (PE; all BD PharMingen, San Diego, CA, unless otherwise indicated), human CD34 conjugated to PE, human CD105 conjugated to PE, human CD166 conjugated to PE, human CD309 conjugated to PE, and human CD45 conjugated to fluorescein isothiocyanate (FITC). EPCs used in this study were between passage 6 and 9.

Cell Culture and Treatment of SAC

A stock SAC solution was prepared in ddH₂O at a concentration of 400 mM. Human EPCs cultured in complete, antibiotic-free MCDB-131 medium with 10% heat-inactivated FBS, 2 mM L-glutamine, and 1.5 g/L sodium bicarbonate in tissue culture dishes coated with type I collagen (50 µg/mL). The EPC monolayers were incubated with SAC at various concentrations (0, 10, 100, and 250 µM) at 1 or 8 h time points.

Assessment of Cell Proliferation

The 3-[4,5-Dimethylthiazolyl]-2,5-diphenyltetrazolium bromide (MTT) assay was performed to detect the cell proliferation. EPCs were seeded in 24-well plates, each well containing 1×10^5 cells. The culture medium was replaced by media in which there were SAC in the presence or absence of different inhibitors for 6 h, respectively. There were triplicate tests for each inhibitor. At the end of the experiment, one of the plates was taken out and fresh MTT (final concentration 0.5 mg/mL in PBS) was added to each well. After 2 h incubation, the culture media were discarded, 200 µL of acidic isopropanol were added to each well and vibrated to dissolve the depositor. The optical density was measured at 570 nm with a microplate reader (Tecan, Männedorf, Switzerland).

Protein Extraction and Western Blotting Analysis

Protein extraction was prepared by using an NE-PER commercial kit with inhibitors against protease and phosphatase. Cell cultures were centrifuged for 10 min at 12,000× *g* to separate the supernatant fraction (cytoplasmic extract) from the remaining nuclear proteins. There was no contamination between the cytoplasmic and nuclear fractions. Cytoplasmic proteins (60 µg) were fractioned using 10% sodium-dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a PVDF membrane. After the protein separation, PVDF membrane was blotted with specific antibodies against target protein such as c-kit. The blots were washed and reprobed with antibody against internal control actin protein. The remaining proteins such as p-Akt (T308), p-GSK-3β (S9), p-eNOS(S1177), t-Akt, t-GSK-3β, and t-eNOS in cell extracts were detected using protocols similar to those previously described.²⁹ The corresponding t-Akt, t-GSK-3β, and t-eNOS proteins were used as loading controls for p-Akt (T308), p-GSK-3β (S9), and p-eNOS(S1177) proteins, respectively. Actin was used as an internal control for the expression of cytoplasmic proteins. Histone H3 was used as a negative internal control for the expression of cytoplasmic proteins.

Nuclear proteins (60 µg) were also measured using the same process. After transferring the protein to the PVDF membrane, the membrane was blotted using a monoclonal antibody against target β-catenin. The blots were stripped and reprobed with internal control Lamin A antibody. Nuclear cyclinD1 and CDK4 proteins were measured using the same procedure.

Neovascularization Assay

Fifty microliter aliquots of Matrigel (4 mg/mL) were placed in 96-well cell culture plates until gelatinization at 37 °C. For the neovascularization assay, EPCs were cultured in the presence or absence of SAC in 96-well plates (1×10^4 cells/well) using complete MCDB-131 medium. To examine the molecular mechanisms of action, EPCs were cultured in the presence or absence of SAC in the presence of signaling pathway inhibitors including wortmannin, PD098059, or L-NAME. After seeding in cell culture plates for 8 h, images of neovascularization in EPCs were documented under an inverted phase-contrast microscopy at a magnification of 40× with an Olympus Imaging System (Tokyo, Japan) and analyzed by using National Institutes of Health (NIH)-image analyzer software program (Scion Corp., Frederick, MD, USA). Neovascularization index was defined as the ratio of total length of neovascularization in various subgroup to the one in control subgroup.

Xenograft Implantation of EPCs

The animal protocol was reviewed and approved by an ethics committee and the Institutional Animal Care and Use Committee (IACUC) at China Medical University (animal protocol no. 101-134-N). Female adult BALB/cAnN.Cg-Foxn1nu/CrlNarl (BALB/c) AnN-Foxn1 immunodeficient nude mice (18 to 23 g) approximately 3 to 4 wk old were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were kept in a specific pathogen-free facility in accordance with standard regulations and principles. A neovascularization xenograft model in experimental mice was created by subcutaneous injection of EPCs (3×10^6 EPCs/ per mouse) in phosphate buffer saline (PBS) and Matrigel (0.15 mL) in each BALB/c nude mouse. After inoculation of the EPCs, these experimental mice were divided into 4 subgroups (N = 4), and 5 mice per subgroup (n = 5). During the entire period of the experiment, the mice were fed an AIN-93 M diet. The positive control group had an injection of EPCs with a Matrigel mixture and SCF (20 ng/mL). In the negative control group, mice were injected with a mixture of Matrigel and EPCs only. SAC was dissolved in ddH₂O and stored in the freezer at −0 °C. For the experimental groups, SAC was provided to mice by gavage feeding at a dosage of 0.2 or 2 mg/kg of body weight (BW) per day after inoculation with EPCs. These experimental mice were sacrificed after being euthanized with CO₂ inhalation at the end of second week. The Matrigel inoculated for neovascularization was detached from the nude mice for further investigation.

Mouse Ischemic Hind-limb Model

Nine-week old female C57BL mice (n = 6 per subgroup) were randomly distributed into 3 subgroups (N = 3): the control subgroup receiving ddH₂O only and experimental

low SAC or high SAC subgroup receiving SAC (at 0.2 or 2 mg/kg of BW per day) by gavage feeding. A microsurgery of the hind-limb ischemia model was performed by a removal of the left femoral artery according to the previous protocol.⁴³ Images of blood recovery were documented with a Laser Doppler Perfusion (LDP) imaging system after the surgery and then documented daily. After completion of image monitoring, the mice were sacrificed at the end of second week. The ischemic limb tissues were removed from mice and frozen immediately.

Histopathological, Immunohistochemical, and Immunofluorescent Staining of Neovasculogenesis

Frozen liver tissues were cut in 5- μ m sections and immediately fixed with 4% paraformaldehyde. Sections were stained with Meyer's hematoxylin-eosin staining for light microscopy. Liver tissues were examined in a blinded manner per tissue section and documented at 200 \times magnification in any of the mice receiving SCF (20 ng/mL) or SAC (either 0.2 or 2 mg/kg of BW).

For immunohistochemistry analysis, frozen tissue section was treated with 0.3% H₂O₂ to inactivate the intrinsic peroxidase. Nonspecific protein reaction was suppressed with 10% normal serum for 1 h. Frozen tissue sections were further incubated with specific antibody (at 1:300 ratio dilution) against target protein such as c-kit. After the antibody incubation, tissue sections were rinsed with PBS and treated with biotinylated immunoglobulin G at room temperature for 1 h. The final reaction was completed by staining with avidin-biotin complex reagent, diaminobenzidine, and H₂O₂.

For immunostaining, frozen tissues were sectioned and probed with specific antibodies against target proteins such as c-kit and VE-cadherin. The sectioned tissues treated with anti-VE-cadherin primary antibody were then exposed to a secondary antibody with an FITC. Other sectioned tissues against anti-c-kit antibody were probed with a TxRd-labeled secondary antibody. The location of nuclei in these sectioned tissues was determined by staining with 4,6-diamidino-2-phenyl indole. Imaging was performed at 600 \times magnification. All images were documented with an Olympus BX-51 microscope using an Olympus imaging system at 200 \times magnification.

Statistical Analysis

SYSTAT biostatistic software, Version 11 (Chicago, IL, USA) was used to determine the differences between the experimental (SAC treatment) and control groups of EPCs. Statistical difference in the levels of neovasculogenesis is required to reject the null hypothesis of no difference between the mean values from experimental and control subgroups by using 1-way analysis of variance. A Duncan's multiple range test was used to evaluate differences among different subgroups. For the analysis of Western blotting results, a statistical difference between control and

experimental subgroups was examined using the Student's *t*-test at the *P* = 0.05 significance level.

Results

SAC Effectively Induces Neovasculogenesis of EPCs In Vitro

To investigate the in vitro neovasculogenic effects of SAC in EPCs, tubular formation analysis was performed. In this study, SCF was selected as a positive control group for neovascularization. The results show that SCF significantly induced neovasculogenesis (Fig. 1B). In comparison with the untreated control subgroup, SCF at concentrations of 10 and 20 ng/mL significantly induced neovasculogenesis of EPCs up to 1.36- and 1.42-fold, respectively (*P* < 0.05). Treatment with SAC also significantly induced neovasculogenesis of EPCs in vitro (Fig. 1B; *P* < 0.05). Compared to the control group, SAC at concentrations of 10, 100, and 250 μ M significantly increased neovasculogenesis by approximately 1.2-, 1.3-, and 1.39-fold (*P* < 0.05), respectively. The results show that SAC effectively induced neovasculogenesis of EPCs in vitro.

SAC Modulates Neovasculogenesis through PI3-K/Akt/eNOS Signaling Pathways in EPCs

We used various inhibitors of the PI3-K and eNOS proteins to examine the relevant signaling cascades involved in the molecular mechanisms underlying the SAC-mediated augmentation of neovascularization in EPCs. As shown in Fig. 2A, treatment with SAC alone (treatment subgroup; panel b) significantly induced the neovasculogenesis of EPCs compared to the untreated subgroup (Fig. 2A, panel a). Moreover, treatments with wortmannin (a PI3-K inhibitor; panel d) or L-NAME (an eNOS inhibitor; panel f) significantly inhibited SAC-mediated neovasculogenesis in EPCs (*P* < 0.05). Compared to the SAC-treated subgroup (panel b), L-NAME (0.3 mM) inhibited SAC-mediated neovasculogenesis up to 25%. Wortmannin (10 μ M), on the other hand, inhibited SAC-mediated neovasculogenesis up to levels as high as 71% that of the SAC-treated subgroup. However, wortmannin alone (panel c), but not L-NAME alone (panel e), significantly inhibited neovasculogenesis in EPCs (Fig. 2B). Neither wortmannin nor L-NAME had any cytotoxic effects on EPCs (Fig. 2C). These results suggest the PI3-K/Akt/eNOS signaling pathways are involved in SAC-mediated neovasculogenesis. The inhibitory effects of wortmannin and L-NAME on neovasculogenesis were not due to cytotoxic effects.

SAC Augmented c-Kit Expression and Induced Phosphorylation of the Akt/eNOS Signaling Proteins in EPCs

As shown in Fig. 3, SAC significantly augmented c-kit protein expression (*P* < 0.05). Furthermore, SAC significantly

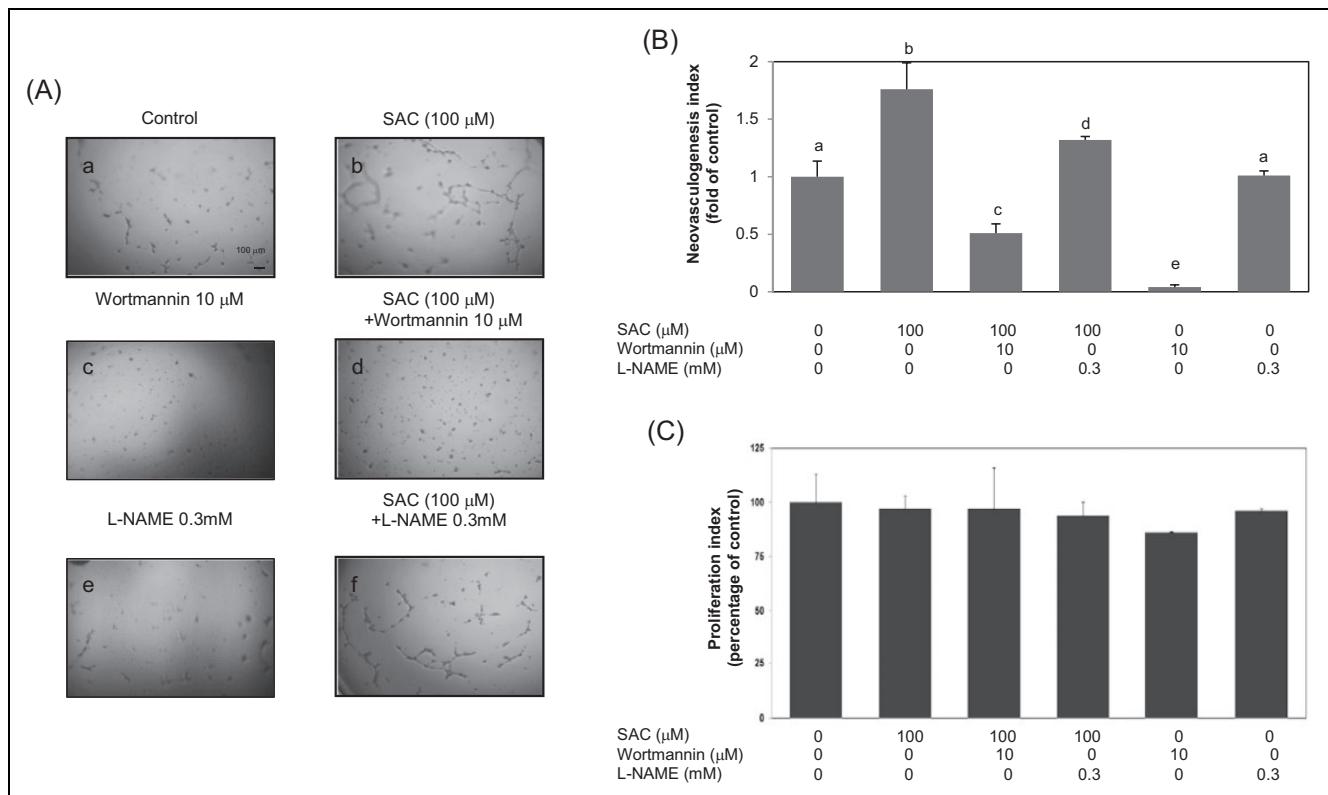


Fig. 2. S-allylcysteine (SAC) modulates neovascuogenesis through PI3-K/Akt/endothelial nitric oxide synthase signaling pathways in endothelial progenitor cells (EPCs). (A) EPCs were treated with wortmannin (10 μ M; panel c, d) or L-NAME (0.3 mM; panel e, f) in the absence or presence of 100 μ M of SAC until the analysis of neovascularization for 8 h, respectively. (B) The values (mean \pm standard deviation [SD]) are represented as neovascuogenesis index (folds of tube formation in comparison with the untreated control group) in 8 randomly selected fields in each culture dish. A different letter represents a statistically significant difference among different subgroups ($P < 0.05$). (C) Cell viability was measured by using 3-[4,5-dimethylthiaol]-2,5-diphenyltetrazolium bromide analysis. The proliferation index is represented as the mean \pm SD. No significant difference was found ($P > 0.05$).

enhanced the phosphorylation of the Akt protein and downstream target molecules such as GSK-3 β and eNOS proteins in EPCs ($P < 0.05$). These results suggest that the SAC-mediated phosphorylation levels (i.e., activation) of the Akt, eNOS, and GSK-3 β proteins are dependent on upregulation of c-kit protein expression.

SAC Upregulated the Expression of Nuclear β -catenin, Cyclin D1, and CDK4 Proteins in EPCs

We examined whether SAC regulated the downstream nuclear proteins in EPCs to further investigate the mechanisms of action underlying the effect of SAC. As shown in Fig. 4, SAC significantly induced the expression of β -catenin, cyclin D1, and CDK4 proteins in EPCs. These results are in agreement with the previously reported evidence⁴⁴ and suggest that the SAC-mediated phosphorylation of GSK-3 β contributes to the upregulation of β -catenin and cyclin D1 proteins in EPCs. Therefore, these results suggested that SAC functions as a neovascuogenic phytochemical, in part, through an upregulation of β -catenin, cyclin D1, and CDK4 proteins.

SAC Consumption Induced the Neovascuogenesis and Increased c-Kit Protein Level in Animal Models

To investigate the conclusion drawn from our in vitro results, we further examined whether SAC consumption would induce neovascuogenesis in mice. In the xenograft model of neovascuogenesis, mice were given SAC (0.2 or 2 mg/kg of BW per day) for 2 wk. The control group received ddH₂O without SAC. As in the case of the in vitro EPC study above, the SCF-treated mice were used as the positive control subgroup for neovascularization (Fig. 5A, B). SAC consumption (2 mg/kg of BW per day) significantly augmented the neovascularization in the EPC-inoculated mouse model of neovascuogenesis. Moreover, SAC consumption significantly increased the amount of c-kit expressing EPCs in the experimental animals (Fig. 5C). These results demonstrate that SAC consumption is able to increase c-kit expression and neovascularization in a mouse xenograft model. Taken together, these results support the notion that SAC consumption induces an increase in the c-kit protein level and EPC-mediated neovascularization in experimental animals. No hepatotoxicity was observed in any of the mice receiving SAC at either 0.2 or 2 mg/kg of BW (Fig. 5D).

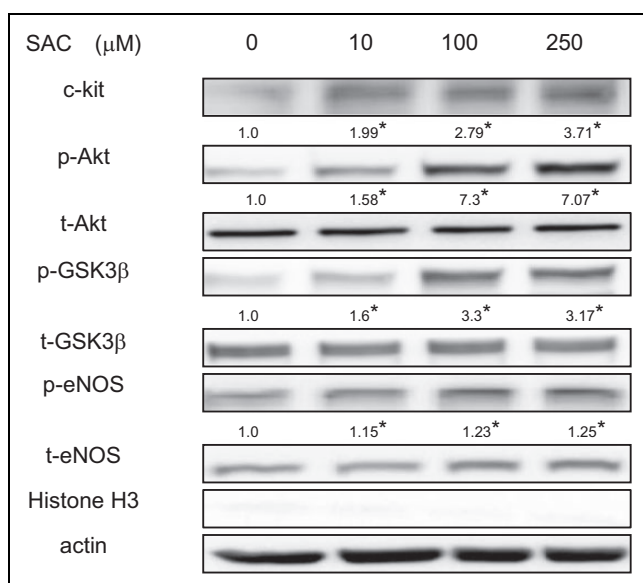


Fig. 3. S-allylcysteine (SAC) augmented c-kit expression and induced phosphorylation of the Akt/endothelial nitric oxide synthase (eNOS) signaling proteins in endothelial progenitor cells (EPCs). EPCs were treated with SAC (10, 100, and 250 μM). Cytoplasmic proteins were measured for the detection of c-kit, p-Akt, t-Akt, p-GSK-3β, t-GSK-3β, p-eNOS, t-eNOS, and actin proteins by using Western blotting analysis. The levels of detection represented the total protein of c-kit or the phosphorylation level of Akt, GSK-3β, and eNOS proteins in the cytoplasm fractions in triplicate. The integrated densities of these proteins were analyzed using image analysis software (BioRad Inc.) and adjusted with the corresponding control proteins such as actin, t-Akt, t-GSK-3β, and t-eNOS, respectively. Histone H3 nuclear protein was used as a negative internal control. The asterisk represents a statistically significant difference comparing to control subgroup ($P < 0.05$).

SAC Consumption Exerted Protective Effects against Ischemic Injury via an Augmentation of Neovascularization and Collateral Blood Flow Recovery in Mice

Finally, we examined whether SAC would augment recovery from ischemic injuries through neovascularogenesis in an ischemic animal model. A mouse model of ischemic injury was performed by ischemic microsurgery of the unilateral hind limb in C57/BL mice ($n = 6$ for each subgroup). Mice were divided into a control subgroup with ddH₂O feeding only and experimental subgroups with SAC feeding by gavage at 0.2 mg/kg (low SAC) and 2 mg/kg (high SAC) of BW/per day. As shown in Fig. 6A, the control mice exhibited slow blood flow recovery after ischemic microsurgery as shown by laser Doppler imaging. SAC consumption (at dosages of 0.2 or 2 mg/kg of BW per day) significantly improved blood flow recovery in ischemic surgery mice. The effect in the high SAC (2 mg/kg of BW) seemed to be more effective than the low SAC subgroup (0.2 mg/kg of BW) on the augmentation of blood flow recovery in ischemic tissues ($P < 0.05$; Fig. 6B). Immunofluorescent staining indicated that both the low SAC and high SAC subgroups displayed

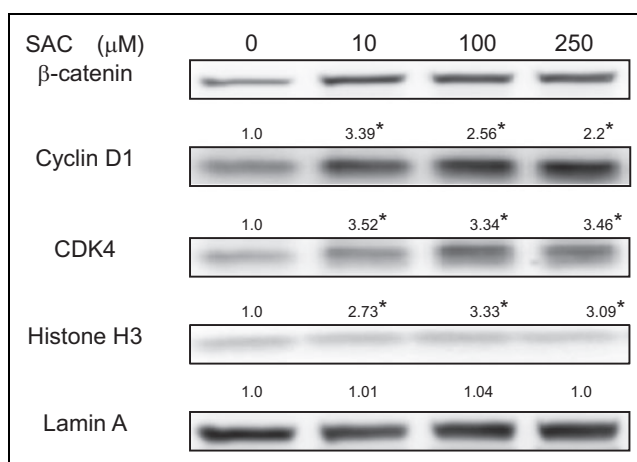


Fig. 4. S-allylcysteine (SAC) upregulated the expression of nuclear β-catenin, cyclin D1, and CDK4 proteins in endothelial progenitor cells (EPCs). EPCs were treated with SAC (10, 100, and 250 μM) for 8 h. Nuclear proteins were detected for β-catenin, cyclin D1, CDK4, and lamin A by using Western blotting analysis. The levels of measurement represented the amounts of β-catenin, cyclin D1, and CDK4 in the nuclei of EPCs. The integrated densities of the β-catenin, cyclin D1, and CDK4 proteins were analyzed using image analysis software (BioRad Inc.) and adjusted with the internal control lamin A protein. The asterisk represents a statistically significant difference compared to control subgroup ($P < 0.05$).

significantly increased c-kit expression in vivo (Fig. 6C). The quantitative results demonstrated that the low SAC and high SAC subgroups both exhibited a significantly augmented c-kit protein level in the mice with ischemic injuries (Fig. 6D). SAC consumption, however, did not have any significant effects on VE-cadherin expression ($P > 0.05$; Fig. 6C, D). The results showed that SAC consumption (at a dosage of 0.2 and 2 mg/kg of BW) significantly enhanced the expression of c-kit protein in ischemic tissues. This suggests that SAC is a potential therapeutic agent for neovascularogenesis by its effect on the upregulation of c-kit and assistance given to EPCs to make them better able to migrate naturally into the sites of blood vessels during an ischemic event in vivo.

Discussion

Ischemic injury is an important risk factor for CLI and CAD. Neovascularization in the course of ischemic injury plays a central role in the repair of tissue damage caused by stroke and heart disease. Unlike the case with EPCs, the proliferation and migration of mature ECs result in postnatal angiogenesis from preexisting microcapillaries, thus limiting their potential for tissue repair.⁴⁵ Human hematopoietic EPCs derived from BM, on the other hand, represent a provascularogenic subpopulation that possesses certain critically important characteristics needed for tissue regeneration, such as excellent repair capability and postnatal neovascularization.

These human EPCs are not only similar to angioblasts but also have the potential to differentiate readily into ECs.²⁴

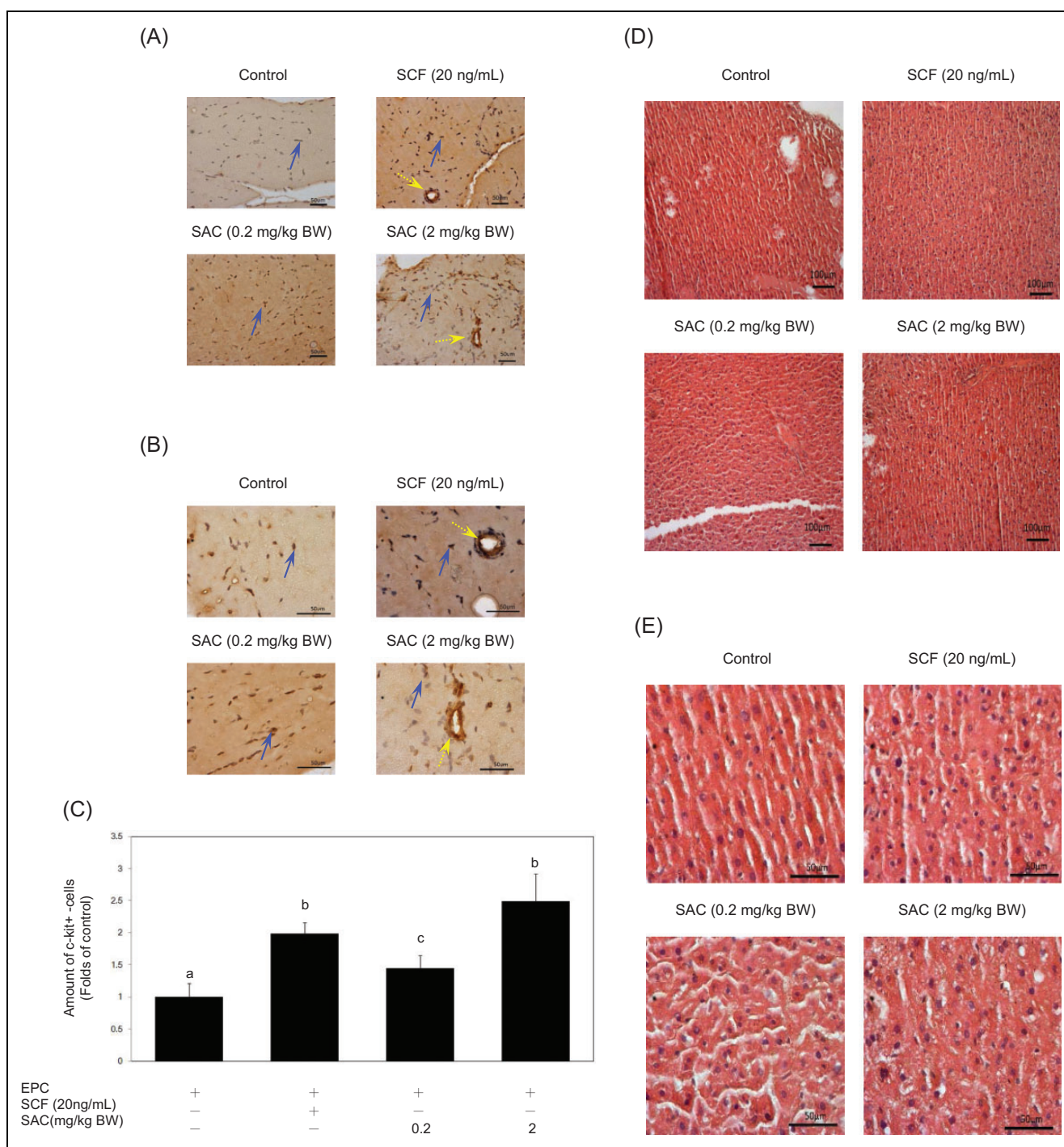


Fig. 5. S-allylcysteine (SAC) consumption induced the neovascularization and increased c-kit protein level in animal models. To develop a negative control subgroup, endothelial progenitor cells (EPCs) alone were transplanted into the nude mice. For the positive control subgroup, EPCs were mixed with stem cell factor (SCF; 20 ng/mL) and subcutaneously inoculated into mice. For the experimental subgroups, nude mice were inoculated with EPCs and received low dosage (low SAC; 0.2 mg/kg of body weight [BW] per day) or high dosage (high SAC; 2 mg/kg of BW per day) of SAC by gavage (oral tube feeding) for 2 wk. The neovascularization samples from experimental animals were sectioned and treated with anti-c-kit antibody by immunohistochemistry staining. Imaging was documented at 200 \times (A) and 400 \times (B) magnification. Intense dark brown indicates the distribution of c-kit protein during neovascularization. The formation of vascular vessel was indicated with a yellow arrow. EPCs were indicated with a blue arrow. The quantitative results (mean \pm standard deviation) of the c-kit $^{+}$ expressing cells were analyzed by using I-way analysis of variance and shown in the bottom panel (C). Different letters indicate a significant difference among different subgroups ($P < 0.05$). Hematoxylin and eosin staining of liver tissues was observed and documented at 200 \times (D) and 400 \times (E) magnification in any of the mice receiving SCF (20 ng/mL) or SAC (either 0.2 or 2 mg/kg of BW).

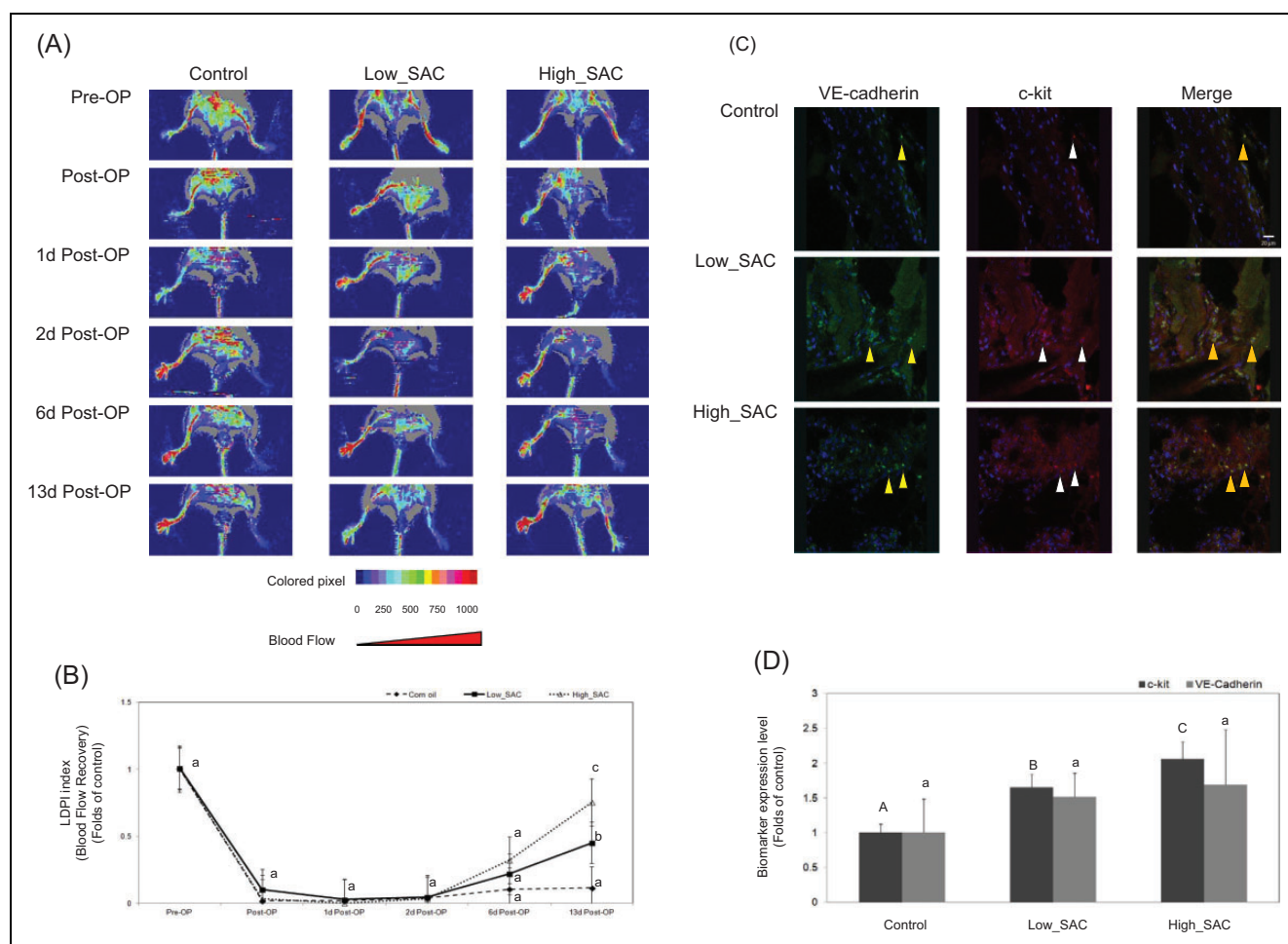


Fig. 6. S-allylcysteine (SAC) consumption exerted protective effects against ischemic injury via an augmentation of neovascularization and collateral blood flow recovery in mice. (A) On the day of operation procedure (OP), mice received the Doppler imaging scanning right before and after the vessel-removal surgery. Experimental mice were given ddH₂O, low dosage (low SAC; 0.2 mg/kg of body weight [BW] per day) and high dosage (high SAC; 2 mg/kg of BW per day) of SAC by gavage (oral tube feeding) for 2 wk after receiving the OP. During the experiment, these C57BL mice received the scheduled scanning by Doppler imaging every day. Quantitative results of blood flow recovery by Doppler imaging were shown in the bottom panel (B). Different letters indicate a significant difference at each stage ($P < 0.05$). (C) Green fluorescence indicates the distribution of VE-cadherin protein stained with the monoclonal antibody (indicted with yellow arrowhead). Red fluorescence indicates the distribution of c-kit protein stained with the monoclonal antibody (indicted with white arrowhead). Yellow fluorescence indicates the codistribution of VE-cadherin/c-kit proteins in neovasclogenesis sites (indicted with orange arrowhead). The blue area represents the location of cell nuclei. Imaging was documented at 600 \times magnification. (D) The values (mean \pm standard deviation) represented the expression of c-kit or VE-cadherin proteins in the bottom panels. Different uppercase letters represent a significant difference of c-kit expression among different subgroups ($P < 0.05$). The same lowercase letter represents a nonsignificant difference of VE-cadherin expression among different subgroups ($P > 0.05$). The intensity of fluorescence was quantified by Image-Pro Plus version 6.0 software (Media Cybernetics, Rockville, MD).

Numerous studies have shown an inverse relationship between EPCs and CAD.⁴⁶ Several clinically approved drugs have been demonstrated to lower the risk of ischemic injury and CAD through an augmentation of circulating EPCs.⁴⁷ However, there are only a few published studies suggesting that dietary factors are able to prevent ischemic injuries and/or CAD.^{29,38} Our previous studies suggested that organosulfides such as DADS- and DATS-augmented neovasclogenesis in vitro.³⁸ In this study, we report the first evidence that SAC acts as an agent for modulating the induction of c-kit-expressing EPCs and hence the augmentation of

neovasclogenesis. Here, the results suggest that SAC prevents ischemic injuries by increased blood flow recovery. Our in vitro results indicate that the neovasclogenetic effects of SAC are comparable to those produced by SCF in EPCs (Fig. 1B). The molecular mechanisms of action underlying the effects of SAC were demonstrated using specific inhibitors against signaling molecules such as Akt and eNOS proteins (Fig. 2). In this study, the results also suggested that SAC induced the neovasclogenesis by modulation of the Akt/eNOS and GSK-3 β signaling cascades (Fig. 3). Several mechanisms might account for the neovasclogenetic effects

of SAC in vitro. One is the augmented phosphorylation of eNOS in EPCs. Studies have shown that the phosphorylation of eNOS leads to increased cell migration and neovascularogenesis.⁴⁸ A previous study suggested that SAC might be involved the reduction of oxidative stress in the atherogenic process.⁴⁰ Although we did not test the antioxidant effects of SAC on neovascularogenesis, the results do suggest an alternative mechanism for neovascularogenesis. This study showed that the beneficial effects of SAC on revascularization of vascular endothelium take place through the activation of the Akt/eNOS signaling cascades. Another study reported that e-NOS/NO may play important roles in the prevention of CVD.³¹ Activation of eNOS proteins has been reported to reduce the senescence of EPCs.⁴⁹ Moreover, SAC appears to prevent myocardial injury by activating eNOS and increasing NO metabolites in the vascular system.^{50,51} These findings are consistent with previous research and support the cardioprotective effects of garlic extracts on the prevention of ischemic injury.

In this study, we provide the first evidence for SAC-induced, EPC-mediated neovascularization. The results show that SAC upregulated c-kit protein expression and increased the activation of the PI3-K/Akt/eNOS signaling cascades. Blocking Akt and eNOS activities with corresponding inhibitors caused a reduction of neovascularogenesis in EPCs. Thus, the augmented expression of c-kit and activation of Akt/eNOS cascades play central roles in the mechanisms of SAC-mediated neovascularogenesis.

Furthermore, the results also indicated that SAC modulated the phosphorylation of multiple signaling molecules such as Akt/GSK-3 β (Fig. 3). Activation of the Akt pathway resulted in the phosphorylation of GSK3 β and led to a reduced phosphorylation level of β -catenin. This might lead to the nuclear translocation of β -catenin. This in turn would upregulate the expression of cyclin D1 and enhance the cell proliferation of EPCs (Fig. 4). This suggests that SAC induced the cell proliferation of EPCs and neovascularogenesis in part through an upregulation of c-kit protein as well as β -catenin and the cyclin D1 proteins.

A mouse xenograft study of neovascularogenesis was utilized to further investigate the in vitro results. SAC enhanced the formation of new blood vessel derived from EPCs (Fig. 5). Furthermore, SAC consumption significantly augmented the c-kit protein expression in these experimental animals. At a dosage of 0.2 or 2 mg/kg of BW per day, SAC significantly induced c-kit protein expression compared with that of the control subgroup (Fig. 5). Moreover, the results showed that consumption of SAC improved blood flow recovery and prevented damage in an ischemic injury animal model (Fig. 6). Taken together, SAC consumption at a dose of 0.2 or 2 mg/kg of BW per day in mice is sufficient to prevent ischemic injuries. It is plausible that SAC consumption alone augments the transplantation effects or enhances the endogenous activity of EPCs. These results suggest that SAC alone or used in cotreatment with EPC transplantation may help prevent ischemic injury and CAD in clinical

applications. Due to the complexity of whole food, it is not accessible to investigate the molecular mechanisms of crude garlic extracts on the prevention of ischemic injury. However, it will be worthy to investigate the preventive application of garlic consumption on the prevention of ischemic injury in further clinical study.

To the best of our knowledge, this is the first evidence showing that SAC, an active organosulfur compound derived from AGE, functions as an effective bioactive phytochemical in the induction of neovascularogenesis, and the prevention of ischemic injuries both in vitro and in vivo.

It is possible that the SAC contained in AGE contributes to the neovascularogenesis of EPCs and the prevention of ischemic injury, although more research is needed to confirm this.

In conclusion, SAC has the potential as a novel neovascularogenic agent that improves blood flow recovery and helps prevent damage from ischemic injuries.

Author's Note

Jia-Ning Syu, Mei-Due Yang, and Shu-Yao Tsai contributed equally to this research work. Any results, conclusions, or implication described in this publication are from the author(s) of this article and do not necessarily reflect the view of the MOE, MOST, National Chung Hsing University, Asia University, University of California, and CMU.

Ethical Approval

This study was approved by an institutional animal care and use committee (IACUC) under protocol No. 104-153-N.

Statement of Human and Animal Rights

We declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research project is supported by the ATU plan supported by the Ministry of Education (MOE); the grant of Ministry of Science and Technology (MOST), Taiwan, under the agreements MOST 101-2320-B-039-054-MY3, 101-2811-B-039-024, 102-2811-B-039-035, 103-2811-B-039-009, 104-2320-B005-010-MY3, 104-2320-B-039-041-MY3, and 104-2911-I005-301 (UCD); Ministry of Education (MOE), Taiwan, under the ATU plan; and China Medical University (CMU) grant under agreements CMU101-Award-10, CMU-100-ASIA-11, CMU-101-ASIA-3, CMU101-S-25, CMU 102-S-05, CMU-102-ASIA-23, CMU-103-S-46, and CMU-104-S-32.

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